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Development of a New Oligonucleotide Array To Identify Staphylococcal Strains at Species Level

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The genus *Staphylococcus* is made up of 36 validated species which contain strains that are pathogenic, saprophytic, or used as starter cultures for the food industry. An oligonucleotide array targeting the manganese-dependent superoxide dismutase (*sodA*) gene was developed to overcome the drawbacks of the conventional methods of identification. Divergences of the *sodA* gene were used to design oligonucleotide probes, and we showed that each of the 36 species had a characteristic pattern of hybridization. To evaluate the array, we analyzed 38 clinical and 38 food or food plant *Staphylococcus* isolates identified by the phenotype-based system VITEK 2 (bioMérieux). This commercial kit failed to identify 8 (21%) of the clinical isolates and 32 (84%) of the food and food plant isolates. In contrast, the oligonucleotide array we designed provided an accurate and rapid method for the identification of staphylococcal strains, isolated from clinical, environmental, or food samples, at species level.

Staphylococci are widely spread in various niches such as clinical environments and food plants. Thirty-six validated described species, including 21 subspecies, belong to the *Staphylococcus* genus according to the *List of Bacterial Names with Standing in Nomenclature*, updated 3 December 2004 (16). Some staphylococcal strains are used for their technological abilities, and others are associated with diseases in humans or animals. *Staphylococcus xylosus* and *S. carnosus* strains are used as starter cultures in fermented meat products, because they contribute to their color and flavor (47). In these products, other staphylococci, such as *S. simulans*, *S. succinus*, *S. equorum*, *S. warneri*, *S. epidermidis*, *S. saprophyticus*, and *S. aureus*, may be found (8), but the last three are also known to be pathogens or opportunistic pathogens. *S. saprophyticus* is the predominant staphylococcal species involved in acute urinary tract infections of young adult women (32). *S. epidermidis* is involved in many infections such as bacteremia and prosthetic and natural valvular endocarditis (50). *S. aureus* is one of the leading causes of food-borne diseases and of nosocomial infections (28, 34).

Because of these yin/yang aspects, much effort has been expended in recent years to identify staphylococci. Several manual and automated methods based on phenotypic characteristics have been developed for identification of the *Staphylococcus* species that are most often isolated from clinical samples (21, 25, 35, 37). Unfortunately, these systems have their limitations, mostly due to phenotypic differences between strains from the same species (33, 37–39). For this reason, methods based on molecular techniques have been developed.

Genus- and species-specific primers have been designed for the identification of bacteria belonging to the genus *Staphylococcus* and for the species-specific detection of *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. xylosus* (2, 18, 31, 33). Some authors associated several genus- and species-specific primer pairs in the same amplification reaction and were able to identify strains at genus level and up to four species (13). These PCR methods are quick and reliable, but they are limited in the number of species that can be identified. Alternative approaches include denaturating gradient gel electrophoresis (8) and sequence determination of the 16S rRNA-encoding gene (*rrs*) (5, 45). However, closely related species may have nearly identical *rrs* sequences, impairing the discriminatory power of these techniques (46). To solve this problem, it is possible to use alternative target genes which exhibit more-divergent sequences than *rrs*. So far the *cpn60* (19), *gla* (53), *femA* (49), *rpoB* (14), and *sodA* (36) genes have been used.

In previous studies, our laboratory reported the sequencing of the *sodA* genes, encoding a manganese-dependent surperoxide dismutase, of *S. xylosus* and *S. carnosus* (3, 4). At the same time, Poyart et al. published the sequences of the *sodA* genes of nearly all known species of staphylococci (36). Pairwise comparison of these sequences revealed a mean identity (81.5%) lower than that calculated for the *rrs* sequences of staphylococci (98%). Therefore, the *sodA* gene will be a more discriminatory target sequence than *rrs* for differentiation of closely related staphylococci. However, the *sodA* sequences of pairs of type strains of subspecies shared more than 99.3% identity and did not allow discrimination at the subspecies level, except for the *sodA* genes of *Staphylococcus cohnii* subsp. *cohnii* and *Staphylococcus cohnii* subsp. *urealyticus*, which display 4% sequence divergence. Furthermore, Poyart et al. demonstrated that the *sodA* sequences of strains of the same species isolated from food or clinical samples displayed less than 1.5% divergence from the sequence of the corresponding type strain (36). In conclusion, they proposed that the sequence

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polymorphism of the *sodA* gene could allow the development of assays based on DNA chip technologies.

The potential for microbial diagnostics of DNA microarrays, originally developed for whole-genome gene expression analyses (42, 48), is very high, since they allow simultaneous product interrogation with a large number of probe sequences (10, 11). Recent studies showed the accurateness of such tools at detecting and identifying a great number of bacteria at the genus or species level in a single assay (15, 51, 52). Despite their very interesting abilities, microarrays are not yet common in microbial diagnostic laboratories. Part of the reason is the considerable initial financial investment. A recent survey conducted by the Association of Biomolecular Resource Facilities (ABRF) Microarray Research Group estimated the mean cost for setting up a microarray facility at $286,000 (22). Similar but less expensive techniques can be used. Oligonucleotide probe sets spotted onto nylon or nitrocellulose membranes have been used for bacterial identification for 10 years. In 1994, Kaufhold et al. used allele-specific oligonucleotide probes fixed to a membrane to rapidly identify strains of group A streptococci (27). Since that preliminary work, other authors have used closely related techniques to identify bacteria at the genus or species level (6, 40, 41).

In this study, we demonstrated the accurateness of such a tool for identification of staphylococcal strains at species level. This system, which we called "Staph. Array," couples PCR amplification of the *sodA* gene with an oligonucleotide-based array to efficiently discriminate all the 36 validated *Staphylococcus* species and the two subspecies of *S. cohnii*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Staphylococcus* type strains used are listed in Table 1. Strains were grown at 30°C in brain heart infusion broth or on brain heart infusion agar (Difco, Detroit, Mich.), with the notable exception of *S. saccharolyticus*, which was grown anaerobically in a medium containing the following (in grams per liter): casein peptone, 10; meat peptone, 5; yeast extract, 5; L-cysteine HCl, 0.4; glucose, 10; NaCl, 5; thioglycolate, 2 (pH 7.2).

Oligonucleotide probe design. A database of *sodA* gene sequences was constructed, and local BLAST comparisons were done with tools embedded in BioEdit software (23). Alignments were done using the ClustalW (12) service at the public website of the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/). To facilitate the probe design, the alignments were reorganized with the "Multialignment Cleaner" tool of the Annhyb package (http://bioinformatics.org/annhyb/). The hairpin and dimer formation abilities of oligonucleotides were tested with the "Oligo" tools of the same package. Melting temperatures of perfect-match duplexes and those of mismatched nucleotides were predicted by the nearest-neighbor method using MELTING (29). All oligonucleotide probes were synthesized with a 5′-terminal amino group by Operon Biotechnologies (Germany) to allow covalent coupling of probes to the membrane. Probes used in this study are reported in Table 2.

Array preparation. Procedures for covalent coupling of probes followed the protocol described previously, with a Cross-Blot dot blot apparatus (Sebia, France) used instead of a Miniblotter (26). Briefly, a charged nylon membrane (Biodyne C; Pall Biosupport, United Kingdom) was activated for 10 min with freshly prepared 16% (wt/vol) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Across Organics, France). The oligonucleotide probes were applied to the membrane in parallel by using the grid with 34 vertical spacers. After 1 min at room temperature, the membrane was inactivated for 8 min with 100 mM NaOH and then washed with 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA, pH 7.7) (Promega, France) supplemented with 0.1% sodium dodecyl sulfate (SDS; Eurobio Biotechnology, France) for 5 min at 60°C.

Target preparation and hybridization procedures. Primers D_1 and D_2, used to amplify the internal part of the *sodA* gene ($sodA_{int}$), have been described previously (36). D_2 was synthesized with a 5′-terminal digoxigenin group (DIG). Amplifications were done with a GenAmp PCR system 9700 PE thermal cycler (Perkin-Elmer, France) and 25-μl volumes containing 0.8 μM of each primer, 50 μM of each deoxyribonucleoside triphosphate, 1.5 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase in 1× buffer according to the manufacturer's instructions (Promega, France). For efficient amplification from one colony picked up from the agar plate, the following conditions were used: 15 min at 4°C; 5 min at 95°C; 40 cycles of 30 s at 94°C, 1 min at 35°C, and 30 s at 72°C; and a final 2-min hold at 72°C. Relative quantification of the 480-bp amplified fragments was performed by comparison with SmartLadder (Eurogentec, France) after electrophoresis through a 1.5% agarose gel and ethidium bromide staining.

The PCR products at a final concentration of 150 ng/ml in 0.5× SSPE–0.1% SDS were heat denatured and cooled on ice immediately. After 5 min of incubation at room temperature in 50 ml of 0.5× SSPE–0.1% SDS, the membrane was placed in the Cross-Blot dot blot apparatus. The 14 horizontal slots of the grid were filled with the denatured target, and hybridization occurred for 1 h at 50°C on a plane surface. The samples were removed carefully, and slots were filled with a prewarmed (60°C) 2× SSPE–0.5% SDS solution. After aspiration, the membrane was taken from the Cross-Blot dot blot apparatus and washed twice in 70 ml 2× SSPE–0.5% SDS for 15 min at 60°C in a rolling bottle. An additional wash with 70 ml 0.1× SSPE–0.5% SDS occurred for 7 min at room temperature. The hybridized targets were detected with the DIG color detection kit (Roche, France).

Other methods of identification. The ID-GPC card of the VITEK 2 system was used for biochemical identification as recommended by the manufacturer (bioMérieux). Analysis of the results was based on the report provided by the VITEK 2 (version 3.01) computer software. Results with low levels of confidence are indicated.

Multiplex PCRs were performed to check the identification at the genus level

TABLE 1. Type strains used in this study

Species or subspecies	Strain
S. arlettae	CIP 103501^T
S. aureus subsp. *aureus*	CIP 65.8^T
S. auricularis	DSM 20609^T
S. capitis subsp. *capitis*	CIP 81.53^T
S. caprae	DSM 20608^T
S. carnosus	DSM 20501^T
S. chromogenes	CIP 81.59^T
S. cohnii subsp *cohnii*	DSM 20260^T
S. cohnii subsp *urealyticus*	CIP104024^T
S. condimenti	CIP 105760^T
S. delphini	CIP 103732^T
S. epidermidis	DSM 20044^T
S. equorum subsp. *equorum*	DSM 20674^T
S. equorum subsp. *linens*	CIP 107656^T
S. felis	ATCC 49168^T
S. fleurettii	CIP 106114^T
S. gallinarum	CIP 103504^T
S. haemolyticus	CIP 81.56^T
S. hominis subsp. *hominis*	CIP 81.57^T
S. hyicus	DSM 20459^T
S. intermedius	CIP 81.60^T
S. kloosii	DSM 20676^T
S. lentus	CIP 81.63^T
S. lugdunensis	DSM 4804^T
S. lutrae	CIP 105399^T
S. muscae	DSM 7068^T
S. nepalensis	CIP 108211^T
S. pasteuri	CIP 103540^T
S. piscifermentans	CIP 103958^T
S. saccharolyticus	CIP 103275^T
S. saprophyticus subsp. *saprophyticus*	CIP 76.125^T
S. schleiferi subsp. *schleiferi*	DSM 4807^T
S. sciuri subsp. *sciuri*	CIP 81.62^T
S. simulans	DSM 20322^T
S. succinus subsp. *succinus*	CIP 107307^T
S. succinus subsp. *casei*	CIP 107658^T
S. vitulinus	CIP 104850^T
S. warneri	DSM 20316^T
S. xylosus	DSM 20266^T

TABLE 2. Probes

Spacer no.[a]	Name	Intended specificity	Accession no. of target sequence	Probe sequence[b]	Concn of probe (μM)
0	PGxylo2	*S. xylosus*	AJ276960	GCCTGGTTAGTAGTTAATAACGGTAACTT	15
1	PGwarn1	*S. warneri*	AJ343932	AGTGTTCCTTCTGATATTCAAACTGCA	15
2	PGsapro1	*S. saprophyticus*	AJ343925	TGTTCCAGAAAATATTCAAACAGCTGTTCGAAA	10
3	PGsim1	*S. simulans*	AJ343930	AATCCTTTCACCTAACTCTGAAGAGAA	10
4	PGcarn1	*S. carnosus*	AJ295150	GTTCGTAATAACGGTGGTGGACATTTAAAC	3
4	PGfleu1	*S. fleurettii*	AY845223	AATCATCGAAGACATCGTTAAGAATTTAAA	5
5	PGsodAu1	*S. aureus*	AF121672	ATTCTGGGAGTTACTTTCACCAAACT	5
5	PGsodM1	*S. aureus*	Z49245	CAAGGTACCGGAAGCGATGAGGATGTC	5
6	PGepider1	*S. epidermidis*	AJ343906	GTGTGCCATCTAATATTCAAACAGCTGT	10
7	PGsciuri1	*S. sciuri*	AJ343928	TTAAACTCTGTTCCTGATGATATCCG	10
8	PGcaprae1	*S. caprae*	AJ343898	CCTTCTGATATTCAAACAGCAGTACGTAACAATGG	10
9	PGhyicus1	*S. hyicus*	AJ343913	GACCAATTACCTGAGGATAAAAAGACTGCG	10
10	PGvitu1	*S. vitulinus*	AJ343931	AAAAATTTAAATTCTGTTCCTGAAAATATTCGTACTGC	10
11	PGschlei1	*S. schleiferi*	AJ343927	GTGTACCTGAAGATAAACGTACTGAGTTC	10
12	PGgali1	*S. gallinarum*	AJ343909	GAAATCCACCATGGTAAACACCACAACA	5
12	PGnep1	*S. nepalensis*	AY878698	AAGCACCACAAC**G**CTTACGTAACTA	5
13	PGsacch1	*S. saccharolyticus*	AJ343923	GACAATGTCCCATCAAATATTCAAACAGC	10
13	PGcoure1	*S. cohnii* subsp. *urealitycus*	AJ343903	GGCTAGTTGTTAATAATGGCAATTTAGA	5
14	PGfel1	*S. felis*	AJ343908	TTGCCAATGTAGATAGTCTTCCAGAAGA	10
15	PGlutr1	*S. lutrae*	AJ343918	TTTAATCACACATTTAGATCGCGTACCTG	15
16	PGdelph1	*S. delphini*	AJ343905	GTACCAGAAAACTTACGTACAGCAGTTCG	10
17	PGequor1	*S. equorum*	AJ343907	GATGCATTCAAAGAAGAGTTTGCTAACC	10
18	PGpiscif1	*S. piscifermentans*	AJ343921	CCACTCATTATTCTGGCAACTTCTTAC	5
19	PGlentus1	*S. lentus*	AJ343916	TGAACCATCAGGCGAAGTAGTAGATG	10
20	PGkloosi1	*S. kloosii*	AJ343915	TGGTGGGGGACATATTAACCATTCATT	15
21	PGcohnii1	*S. cohnii* subsp. *cohnii*	AJ343902	AGAGTCTAAATCAATTGAAGAAATATTGCAAA	10
22	PGcondi1	*S. condimenti*	AJ343904	TAATGGTGGTGG**G**CATCTAAACCATTCAT	20
23	PGhomi1	*S. hominis*	AJ343911	GTATCTGAAAATATTCAAACAGCAGTACGT	10
24	PGhaemo1	*S. haemolyticus*	AJ343910	TCTGCAGTTGAGGGAACAGATCTT	10
25	PGcapitis1	*S. capitis*	AJ343896	CTGCTGCACGCTTTGGATCTG	10
26	PGarlett1	*S. arlettae*	AJ343894	AATTGAAGAAATCGTCGCTAACTTAGATAGC	10
27	PGauri1	*S. auricularis*	AJ343895	GGGGTTGGCTCGTTGTAAATGCTG	10
28	PGpaste1	*S. pasteuri*	AJ343920	ACCTTCTGATATCCAAACTGCTGTTAGA	10
29	PGchromo1	*S. chromogenes*	AJ343901	AATAGCGTACCAGAAGATAAACAAACTCC	20
30	PGlug1	*S. lugdunensis*	AJ343917	GCCAATTTAGATAGCGTTCCTGAAAACAT	10
31	PGinter1	*S. intermedius*	AJ343914	AAATAGTGTACCTGAAAACATTCGTACAGC	10
32	PGmusc1	*S. muscae*	AJ343919	GATGTACCTGAAGAAAAACGCACAGC	10
33	PGsuc2	*S. succinus*	AY845222	GCGAATAAAGCTGCAGCACGTT	10

[a] Spacer numbers 0 to 33 correspond to the spacer numbers in Table 3 and Fig. 1.
[b] Positions of mismatches with target sequences are indicated by boldfaced characters.

and the identification of *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and *S. xylosus* strains (13).

The internal base compositions of the *sodA* genes were determined using primers D_1 and D_2 as previously described (36). Sequences were compared against a local database of $sodA_{int}$ gene sequences. Identification to the species level was based on ≥97% sequence identity with the type strain sequence and a ≥5% sequence difference from the next closest species.

Nucleotide sequence accession numbers. All partial staphylococcal sequences determined in this study were deposited in GenBank. The accession numbers of the *sodA* sequences of *Staphylococcus succinus* subsp. *succinus*, *Staphylococcus succinus* subsp. *casei*, *Staphylococcus equorum* subsp. *linens*, *S. fleurettii*, and *S. nepalensis* are AY845222, AY842858, AY878697, AY845223, and AY878698, respectively.

RESULTS

Determination of the *sodA* internal gene sequences from type strains of coagulase-negative staphylococci. The $sodA_{int}$ sequences of type strains of *S. equorum* subsp. *linens*, *S. succinus* subsp. *succinus*, *S. succinus* subsp. *casei*, *S. fleurettii*, and *S. nepalensis* were amplified. These fragments were sequenced, and sequence comparisons were done using BLAST (1). The base composition of *S. equorum* subsp. *linens* $sodA_{int}$ was completely identical to that previously published for the *S. equorum* subsp. *equorum* type strain. The $sodA_{int}$ sequences of *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei* differed in only one base pair. The highest sequence similarity values were (i) 93% for *S. succinus* compared to *S. gallinarum*, (ii) 95% for *S. fleurettii* compared to *S. vitulinus*, and (iii) 92% for *S. nepalensis* compared to *S. cohnii* subsp. *urealyticus*.

Design of probes. The available partial sequences of *sodA* and those we determined were used to create characteristic probes for the 36 species. Oligonucleotides of 21 to 38 bases, with predicted melting temperatures from 61°C to 68°C, were chosen from dissimilar parts noted in aligned *sodA* sequences. We rejected sequences with predicted stable hairpins and dimers or with unsatisfactory specificities. A central mismatch was introduced into probes PGcondi1 and PGnep1. These G/T artificial mismatches were created to increase the specificity of these probes. Candidate probes were tested on the array under different conditions, and those that were adopted are reported in Table 2. Probe concentrations were empirically modified to allow better discrimination.

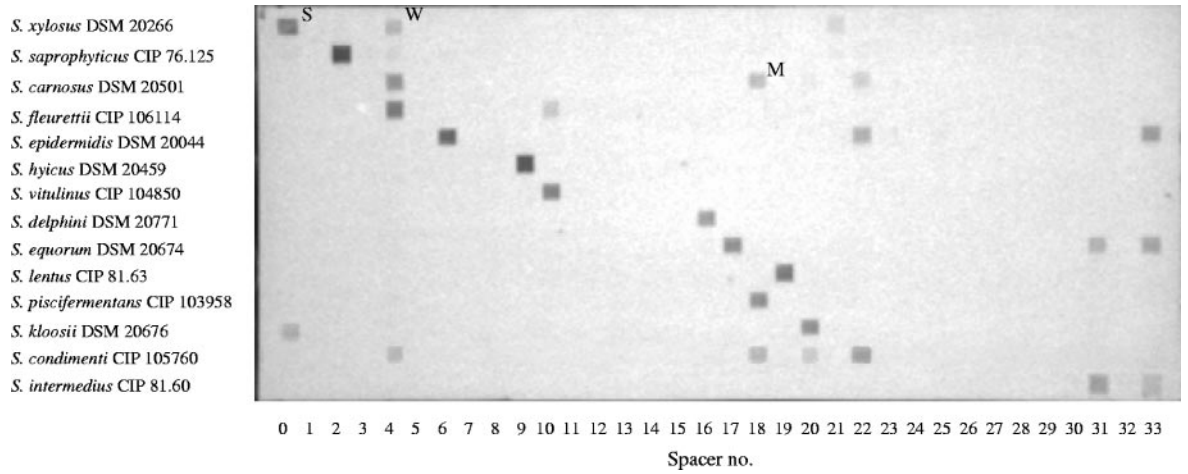

FIG. 1. Hybridization patterns obtained with reference type strains of *Staphylococcus*. Lanes 0 to 33 correspond to the numbers of the spacers in which the probes described in Table 2 have been fixed. Levels of hybridization are indicated as follows: W, weak; M, medium; S, strong.

Validation with type strains. Our array was first tested with type strains of each validated *Staphylococcus* species (Table 1). As expected, not only unique spots but unique patterns of spots were obtained (Fig. 1). This was due to the conditions of hybridization, which allowed some mismatched probe-target pairs to hybridize, i.e., some probes hybridized not only with the targets for which they were designed but also with targets from closely related species. However, comparisons of the patterns of hybridization showed that a unique pattern was found for each species (Table 3). The probes that we designed discriminated targets with differences in their base composition as low as 3%, since we distinguished *S. condimenti* from *S. carnosus* or *S. piscifermentans* and we obtained distinct patterns for the two subspecies of *S. cohnii*. We also discriminated species that are difficult to differentiate on the basis of their *rrs* sequences, such as the *S. intermedius* and *S. delphini* species and the *S. nepalensis* and *S. cohnii* subsp. *urealyticus* species (5, 44). We could not distinguish between the two subspecies of *S. succinus* or the two subspecies of *S. equorum* because their $sodA_{int}$ sequences were identical.

Application to strains isolated from clinical samples. A total of 38 strains (Table 4) from clinical samples were identified first by a phenotypic approach using the VITEK 2 system (bioMérieux), and these results were compared to the array identification. Results were identical for 30 strains of *S. aureus*, *S. capitis*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. warneri*. Six strains (16%) were misidentified by the VITEK 2. Three strains identified as *S. epidermidis* by VITEK 2 were identified as strains of *S. aureus*, *S. warneri*, and *S. capitis* by the array. Two strains identified as *S. epidermidis* by the array were misidentified as *S. warneri* and *S. hominis* by VITEK 2. One strain identified as *S. simulans* by VITEK 2 did not give any hybridization result on the array. Multiplex PCR confirmed that this strain was not a staphylococcus. Two strains (5%) could not be identified by VITEK 2. They were identified as *S. epidermidis* and *S. hominis*.

Application to strains isolated from food or food plant samples. A total of 38 strains (Table 5) from food or food plant samples were also identified by the VITEK 2 system, and the results of the identification were compared to the results from the array. Some species commonly isolated from food or food plants, such as *S. equorum* and *S. succinus* (9), are not included in the VITEK card database; thus, the strains belonging to these species could not be identified by the VITEK 2 system. But even for species included in the ID-GPC database, some misidentification or lack of identification occurred. None of eight *S. xylosus* strains were correctly identified. Five strains were misidentified as *S. saprophyticus*, two were misidentified as *S. cohnii* subsp. *urealyticus*, and one was not identified. Only one of four *S. saprophyticus* strains was correctly identified; the others were either misidentified as *S. chromogenes* or *S. auricularis* or not identified. Of the two strains of *S. epidermidis*, one was correctly identified, while the other was identified as *Kocuria varians*. The *S. warneri* strain was also misidentified as *K. varians*. Strains of *S. hominis*, *S. capitis*, and *S. sciuri* were correctly identified. All the identifications done via the array were confirmed by multiplex PCR when suitable and by sequencing of the $sodA_{int}$ fragments of these strains.

Stability of patterns obtained with wild-type strains. The stability of the patterns of hybridization obtained for wild-type strains was investigated. As an example, Fig. 2 shows the patterns obtained for strains of *S. xylosus* and *S. equorum* isolated from food or food plant samples. Only slight variations in the intensity of hybridization spots occurred. The same stability of hybridization patterns was obtained whatever the origin of the strain (data not shown).

DISCUSSION

In this study, we present a new oligonucleotide array tool, called "Staph. Array," for the identification of the 36 species of staphylococci described and validated and for the discrimination of the two *S. cohnii* subspecies. For this method, universal primers amplifying an internal part of the *sodA* gene were used, followed by hybridization of the denatured PCR products onto an oligonucleotide array.

Because a large amount of *rrs* sequence data is available in a public database, it is not surprising that this gene has been an obvious choice when molecular diagnostic tests based on DNA arrays have been developed. One important drawback of using

TABLE 3. Patterns obtained with type strains of the 36 species of *Staphylococcus*

Species	Hybridization at spacer no.[a]:																																
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
S. arlettae	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	M	−	−	−	−	−	S	−	−	−	−	−	−	S
S. aureus	−	−	−	−	W	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−
S. auricularis	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−
S. capitis	−	W	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−
S. caprae	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−
S. carnosus	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	M	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−
S. chromogenes	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−
S. cohnii subsp *cohnii*	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−
S. cohnii subsp *urealyticus*	−	−	−	−	−	W	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−
S. condimenti	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	M	−	W	−	S	−	−	−	−	−	−	−	−	−	−	−
S. delphini	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. epidermidis	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	W
S. equorum subsp. *equorum*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	M	−	M
S. equorum subsp. *linens*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	M	−	M
S. felis	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−
S. fleurettii	−	−	−	−	S	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. gallinarum	−	−	M	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W
S. haemolyticus	−	−	−	−	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−
S. hominis subsp. *hominis*	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	M
S. hyicus	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. intermedius	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	M
S. kloosii	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−
S. lentus	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. lugdunensis	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W	S	−	−	−	−	−	−	S	M	−	−
S. lutrae	−	−	−	−	−	−	−	−	−	W	−	−	−	−	−	S	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	S
S. muscae	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−
S. nepalensis	−	−	−	−	−	W	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−
S. pasteuri	−	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−
S. piscifermentans	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. saccharolyticus	−	−	−	−	M	−	M	−	M	−	−	−	−	S	−	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−
S. saprophyticus	−	−	S	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. schleiferi	−	−	−	−	M	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. sciuri	−	−	−	−	−	−	−	S	−	−	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. simulans	−	−	−	S	W	−	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. succinus subsp. *succinus*	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S
S. succinus subsp. *casei*	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	S
S. vitulinus	−	−	−	−	−	−	−	−	−	−	S	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. warneri	−	S	−	−	M	−	−	−	−	−	−	−	M	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S. xylosus	S	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W	−	−	−	−	−	−	−	−	−	−	−

[a] Spacer numbers correspond to the numbers of the spacers in which the probes have been fixed, as reported in Table 2. W, weak; M, medium; S, strong; −, no hybridization. See Fig. 1.

rrs genes is their conservative nature. Takahashi et al. pointed out that closely related species of staphylococci could have nearly identical *rrs* base composition (46), decreasing the discriminatory potential of that gene for staphylococci. To bypass this problem, some authors have used more-divergent genes to identify staphylococcal strains. The *femA* (24, 49), *rpoB* (14), *gla* (53), *cpn60* (19), and *sodA* (36) genes have been used. Array techniques have been used with the *femA* and *cpn60* genes. With *femA* a microarray was developed allowing discrimination of only five *Staphylococcus* species (*S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. saprophyticus*) (24). The use of amplification products of the HSP60-encoding gene (*cpn60*) as probes produced better results; Goh et al. identified strains belonging to 30 species (20). However, their system failed to distinguish *S. intermedius* from *S. delphini* strains and did not identify some other strains, probably because they belonged to species not included in their 30-species panel. We choose *sodA* to develop our tool because sequences of that gene were available for 33 out of the 36 species of staphylococci as opposed to 15 for *femA*, 27 for *rpoB*, and 30 for *cpn60*. In the present work, the available sequence data were completed with the *sodA* partial base composition of the five type strains of *S. equorum* subsp. *linens*, *S. fleurettii*, *S. nepalensis*, *S. succinus* subsp. *succinus*, and *S. succinus* subsp. *casei*. This gene proved to be discriminatory at species level, since the sequences obtained were more than 5% divergent from the other *sodA* sequences present in GenBank. However, the $sodA_{int}$ sequences of the two subspecies of *S. succinus* showed an identical base pair composition, like the $sodA_{int}$ sequences of the two subspecies of *S. equorum*. These results confirmed the lack of discriminatory power of the $sodA_{int}$ sequences at the subspecies level (36).

After initial database screening, oligonucleotide probes were selected on the basis of hybridization results obtained by using reference strains as templates. The conditions of hybridization allowed some mismatched duplexes to form. Consequently, cross-hybridization of several probes with some targets obtained from strains belonging to closely related species

TABLE 4. Results of identification of clinical strains by the VITEK 2 and the Staph. Array system

Isolate no.	Identification[a] by:	
	VITEK 2	Staph. Array
S04-059	*S. simulans*	Nonstaphylococcal
S04-026	*S. aureus*	*S. aureus*
S04-027	*S. aureus*	*S. aureus*
S04-029	*S. aureus*	*S. aureus*
S04-031	*S. aureus*	*S. aureus*
S04-032	*S. aureus*	*S. aureus*
S04-040	*S. aureus*	*S. aureus*
S04-041	*S. aureus*	*S. aureus*
S04-042	*S. aureus*	*S. aureus*
S04-054	*S. epidermidis*	*S. aureus*
S04-033	*S. epidermidis*	*S. capitis*
S04-048	*S. capitis*	*S. capitis*
S04-035	*S. epidermidis*	*S. epidermidis*
S04-022	*S. epidermidis*	*S. epidermidis*
S04-023	*S. epidermidis*	*S. epidermidis*
S04-024	*S. epidermidis*	*S. epidermidis*
S04-036	*S. epidermidis*	*S. epidermidis*
S04-028	*S. epidermidis*	*S. epidermidis*
S04-030	*S. epidermidis*	*S. epidermidis*
S04-037	*S. epidermidis*	*S. epidermidis*
S04-034	*S. hominis*	*S. epidermidis*
S04-038	*S. epidermidis*	*S. epidermidis*
S04-055	*S. warneri*[L]	*S. epidermidis*
S04-058	Unidentified	*S. epidermidis*
S04-056	*S. epidermidis*	*S. epidermidis*
S04-057	*S. epidermidis*	*S. epidermidis*
S04-043	*S. haemolyticus*	*S. haemolyticus*
S04-044	*S. haemolyticus*	*S. haemolyticus*
S04-046	*S. haemolyticus*	*S. haemolyticus*
S04-047	*S. haemolyticus*	*S. haemolyticus*
S04-051	*S. haemolyticus*	*S. haemolyticus*
S04-053	*S. haemolyticus*	*S. haemolyticus*
S04-025	*S. hominis*	*S. hominis*
S04-049	Unidentified	*S. hominis*
S04-050	*S. hominis*[L]	*S. hominis*
S04-052	*S. hominis*	*S. hominis*
S04-039	*S. epidermidis*	*S. warneri*
S04-045	*S. warneri*	*S. warneri*

[a] L, identified at a low level of confidence.

TABLE 5. Results of identification of food or food plant strains by the VITEK 2 and the Staph. Array system

Isolate no.[a]	Identification[b] by:	
	VITEK 2	Staph. Array
CIT S03-0458	*Kocuria rosea*	*S. arlettae**
CIT S03-0258	*S. capitis*	*S. capitis*
CIT S03-0356	*K. rosea*[L]	*S. carnosus**
CIT S03-0354	*K. rosea*[L]	*S. carnosus**
CIT S03-0437	*Kocuria varians*	*S. epidermidis*
CIT S03-0438	*S. epidermidis*[L]	*S. epidermidis*
CIT S03-0631	Unidentified	*S. equorum**
CIT S03-0632	*S. cohnii* subsp. *urealyticus*	*S. equorum**
CIT S03-0203	*S. cohnii* subsp. *urealyticus*[L]	*S. equorum**
CIT S03-0204	*S. cohnii* subsp. *urealyticus*	*S. equorum**
CIT S03-0205	*S. cohnii* subsp. *urealyticus*	*S. equorum**
CIT S03-0215	*S. cohnii* subsp. *urealyticus*[L]	*S. equorum**
CIT S03-0214	*K. varians*[L]	*S. equorum**
CIT S03-0190	*K. varians*	*S. equorum**
CIT S03-0670	*S. kloosii*	*S. fleurettii**
CIT S03-0417	*S. hominis*[L]	*S. hominis*
CIT S03-0370	*S. capitis*	*S. pasteuri**
CIT S03-0429	*S. warneri*	*S. pasteuri**
CIT S03-0199	*S. saprophyticus*	*S. saprophyticus*
CIT S03-0451	*S. chromogenes*[L]	*S. saprophyticus*
CIT S03-0480	Unidentified	*S. saprophyticus*
CIT S03-0481	*S. auricularis*[L]	*S. saprophyticus*
CIT S03-0027	*S. sciuri*	*S. sciuri*
CIT S03-0630	*S. kloosii*[L]	*S. succinus**
CIT S03-0657	*S. kloosii*[L]	*S. succinus**
CIT S03-0740	*S. xylosus*[L]	*S. succinus**
CIT S03-0749	*S. kloosii*[L]	*S. succinus**
CIT S03-0579	*S. cohnii* subsp. *cohnii*[L]	*S. vitulinus**
CIT S03-0586	*S. cohnii* subsp. *cohnii*[L]	*S. vitulinus**
CIT S03-0406	*K. varians*[L]	*S. warneri*
CIT S03-0519	*S. saprophyticus*	*S. xylosus*
CIT S03-0520	*S. cohnii* subsp. *urealyticus*[L]	*S. xylosus*
CIT S03-0523	*S. saprophyticus*	*S. xylosus*
CIT S03-0525	*S. saprophyticus*	*S. xylosus*
CIT S03-0526	*S. saprophyticus*	*S. xylosus*
CIT S03-0527	Unidentified	*S. xylosus*
CIT S03-0063	*S. cohnii* subsp. *urealyticus*	*S. xylosus*
CIT S03-0179	*S. saprophyticus*	*S. xylosus*

[a] CIT, Collection INRA de Theix.
[b] L, low level of confidence. *, species not included in the ID-GPC database of VITEK 2.

was observed. However, a unique pattern of hybridization was obtained for each staphylococcal species, allowing us to identify unknown strains. We used the "Staph. Array" system to identify 76 strains from clinical, food, or environmental food samples, and these identifications were compared with those obtained by the VITEK 2 system. VITEK 2 is one of the laboratories' routine identification systems and has been shown to provide reliable results compared to other systems based on phenotypic identification (17, 30). Nineteen species commonly encountered in clinical isolates are included in the ID-GPC database of the VITEK 2 system. We showed that strains can be misidentified or not identified by the commercial system, even strains of species whose identification is covered by the VITEK 2 database; this is especially true for strains isolated from food or food plants. Strangely, identifications with good or higher levels of confidence were obtained when some strains belonging to species not included in the ID-GPC database were submitted to the VITEK 2 system (Table 5). Therefore, caution should be taken when VITEK 2 is used with unsuitable species. The problem of identification by the VITEK 2 system could be explained by the intraspecies variability of the phenotypic traits. In contrast to their phenotypic traits, the patterns obtained with "Staph. Array" were stable for strains belonging to the same species, whatever their origins. This result confirmed the low intraspecies variation of the $sodA_{int}$ sequences (36, 43).

Recent studies have shown that molecular methods give more-accurate results than kits based on biochemical assays (5, 43). Interestingly, one of these studies used *sodA* sequencing to identify staphylococcal strains from clinical isolates (43). Identification was based on 97% sequence identity with the type strain sequence and a 5% difference in sequence from the next closest species. However, these criteria did not allow discrimination of *S. condimenti* and *S. carnosus* (96.7% nucleotide identity) or of *S. condimenti* and *S. piscifermentans* (95.6% nucleotide identity). With "Staph. Array," the hybridization profiles of *S. carnosus*, *S. piscifermentans*, and *S. condimenti* were clearly distinct, so these three species can be identified (Fig. 1).

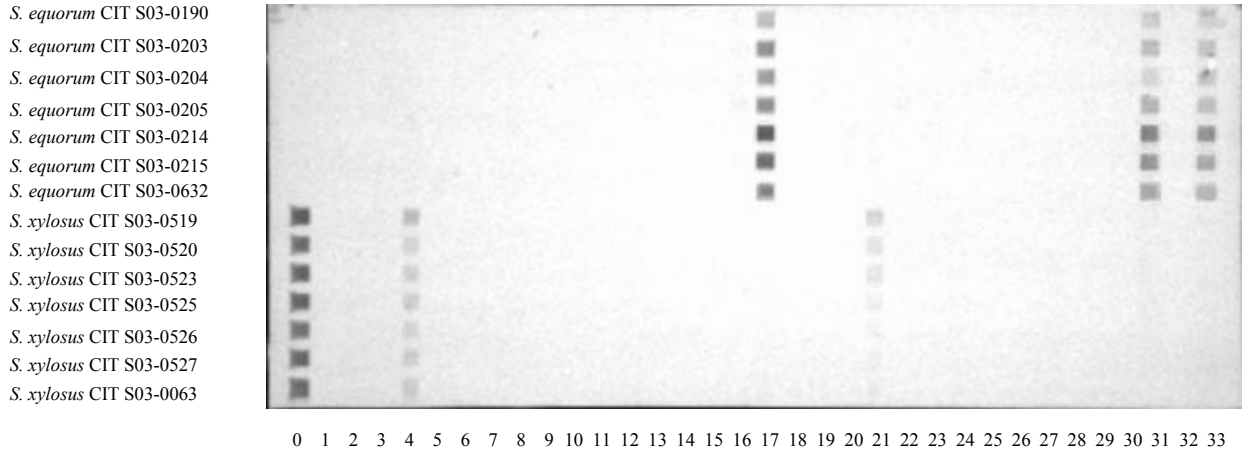

FIG. 2. Hybridization patterns obtained with wild-type strains. Lanes 0 to 33 correspond to the numbers of the spacers in which the probes described in Table 2 have been fixed.

The specificity of the probes we designed was ensured by comparison with the sequences available in GenBank (7). To date, screening of GenBank revealed that the *sodA* gene is present in about 30 bacterial genera, but most of the data covered strains of *Staphylococcus*, *Enterococcus*, *Streptococcus*, *Pasteurella*, and *Mycobacterium*. We cannot exclude the possibility that yet unknown targets hybridize to our probes, but it is unlikely that targets from nonstaphylococcal strains produce patterns of hybridization that can be confused with staphylococcal patterns.

In conclusion, the tool "Staph. Array" allowed the rapid (less than 24 h) and accurate identification of staphylococcal strains at species level. It is the only tool described to date that distinguishes in one shot the 36 validated staphylococcal species and also discriminates the two subspecies of *S. cohnii*.

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